I. AMENDMENTS

A. In the Specification

Please enter rewritten paragraph 11 on page 3 to read as follows:

A1

Figure 3 shows a schematic drawing of a regulatory network associated with a reaction in a metabolic network. Integration of a stoichiometric model and a logical model is achieved through regulatory restraints (logic values of reaction processes) which are used to refine appropriate reaction constraints in the model. If rxnLogic = 1 then use Activity constraints; If rxnLogic = 0 then use Inactivity constraints. Activity constraints set for rxn_{stoich}: (lower bound = 0, upper bound = INF or #); Inactivity constraints for rxn_{stoich}: (lower bound = 0, upper bound = 0). Logic functions: a_1 = (activator/inhibtor) • TF; $a_2 = 1$; c_1 = TF* • pr_1 • *gene1* • *gene2;* $c_2 = pr_3$ • *gene3;* $1_1 = M_{gene1;}$ $1_2 = M_{gene2;}$ $1_3 = M_{gene3}$; p1 = P_{gene1}; • P_{gene2} • $P_{gene3;}$ rsn_{Logic} = Protein • Cofactor • $Substrate_1$ • $Substrate_2$. Time delays can be specified for the switching of each memorization variable after a triggering change in the associated function.

Please enter rewritten paragraph 15 on page 4 to read as follows:

A2

Figure 7 shows a schematic drawing of a simplified core metabolic network. Table 4 provides the stoichiometry of the 20 metabolic reactions included in the network.

Please enter rewritten paragraph 16 on page 4 to read as follows:

Figure 8 shows, in Panel A, a simulation of aerobic growth of *E. coli* on acetate with glucose reutilization; and in Panel B, *in silico* arrays showing the up- or down-regulation of selected genes, or activity of regulatory proteins, in the regulatory network. Panel A shows three time plots showing experimental data (closed squares or triangles) and the corresponding simulations performed using the combined regulatory/metabolic model (solid lines) as well as the stand-alone metabolic model (dashed lines). Table 5 provides parameters used to generate the plots in Panel A.

Please enter rewritten paragraph 17 on page 4 to read as follows:

Figure 9 shows, in Panel A, a simulation of anaerobic growth of E. coli on glucose; and in Panel B, in silico arrays showing the up- or down-regulation of selected genes, or activity of regulatory proteins, in the regulatory network. Table 6 provides the parameters used to generate the plots in Panel A.

Please enter rewritten paragraph 18 on page 4 to read as follows:

Figure 10 shows, in Panel A, a simulation of aerobic growth of E. coli on glucose and lactose; and in Panel B, in silico arrays showing the up- or down-regulation of selected genes, or activity of regulatory proteins, in the regulatory network. Table 7 provides the parameters used to generate the plots of Panel A. Panel A shows time plots showing experimental data (triangles) and the corresponding simulations performed using the combined regulatory/metabolic model (thick solid lines), the stand-alone metabolic model (dashed lines), and the kinetic model described in Kremling (*Metabolic Eng.* 3:362-379 (2001)) (thin solid line).

A2 Cont'd

Please enter rewritten paragraph 139 on page 48 to read as follows:

A3

A skeleton of the biochemical reaction network of core metabolism was formulated, including 20 reactions, 7 of which are regulated as shown in Figure 7. This network provided a simplified representation of core metabolic processes including glycolysis, the pentose phosphate pathway, TCA cycle, fermentation pathways, amino acid biosynthesis and cell growth, along with corresponding regulation pathways including catabolite repression, aerobic/anaerobic regulation, amino acid biosynthesis regulation and carbon storage regulation. The skeleton biochemical reaction network was represented as a skeleton combined regulatory/metabolic model where reactions were represented as linear equations of reactants and stoichiometric coefficients and regulation was represented by regulatory logic statements as shown in Table 4. As shown in Figure 7 and Table 4, four regulatory proteins (Rpo2, RPc1,RPh and RPb) regulated 7 of the 20 reactions in the skeletal network and model.

Please enter rewritten paragraph 156 on page 53 to read as follows:

E. coli has been observed *in vivo* to secrete acetate when grown aerobically on glucose in batch cultures; when glucose is depleted from the environment, the acetate is then reutilized as a substrate. Using the combined regulatory/metabolic and stand-alone metabolic models, activity of an aerobic batch culture of *E. coli* on glucose minimal medium was simulated. Panel A of Figure 8 shows three time plots showing experimental data (closed squares) and the corresponding simulations performed using the combined regulatory/metabolic model (solid lines) as well as the stand-alone metabolic model (dashed lines). In the acetate plot, the regulatory/metabolic model predictions differed from that of the stand-alone metabolic model, as shown. Table 5 provides the parameters required to generate the time plots where parameters were estimated or obtained from Varma and Palsson Appl. Env. Micro. 60:3724-3731 (1994). The major difference between the combined regulatory/metabolic and metabolic stand-alone simulations is in the delayed reaction of the system to depletion of glucose in the growth medium. The stand-alone metabolic network is unable to account for the delays associated with protein synthesis.

A4

Please enter rewritten paragraph 159 on page 54 to read as follows:

The *in silico* models were used to simulate anaerobic growth on glucose, the results of which are shown in Figure 9 which was generated using the parameters provided in Table 6. Under these conditions, the stand-alone metabolic model made similar predictions as the combined regulatory/metabolic model, with a notable exception: the combined regulatory/metabolic model was able to make predictions about the use of a particular isozyme. For example, both models require fumarase activity as part of the optimal flux distribution; however, of the two models only the combined regulatory/metabolic model was able to specifically determine that the *fumB* gene product [which as being] is expressed under anaerobic conditions.

A5

Please enter rewritten paragraph 160 on page 55 to read as follows:

Aerobic growth of *E. coli* on glucose and lactose was simulated using the *in silico* models and compared to *in vivo* observations from mixed batch cultures and to results reported for a kinetic model as described in Kremling et al., Metabolic Eng. 3:362-379 (2001). Overall, the combined regulatory/metabolic model predictions were in good agreement with the *in vivo* observations, comparable with the predictions made by the Kremling model, and better than the predictions of the stand-alone metabolic model as shown in Figure 10 which was generated using the parameters provided in Table 7. The deficiencies in the ability of the stand-alone metabolic model to accurately predict the results of this experiment is most likely due to the concurrent uptake of glucose and lactose, resulting in much more rapid depletion of the substrates and a higher growth rate. Interestingly, because of the larger flux of carbon source uptake, the stand-alone metabolic model predicted that *E. coli* growth should be oxygen-, rather than carbon-limited in this case. Accordingly, the secretion of acetate and formate was predicted by the stand-alone metabolic model. In contrast, the combined regulatory/metabolic model predicted that no secretion will occur under these conditions.

Please enter rewritten paragraph 161 on page 55 to read as follows:

The *in silico* arrays for the simulation (Figure 10B) showed one shift in gene expression, occurring just under five hours. The up-regulation of the lactose uptake and degradation machinery, together with key enzymes in galactose metabolism, enables the system to use lactose as a carbon source once the glucose in the medium has been depleted.

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Cont

Please add the following:

After the second page of Table 3 on page 62, please add the following tables:

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Table 4 provides the stoichiometry of the 20 metabolic reactions include in the network of Figure 7.

REACTION	NAME	REGULATION
Metabolic Reactions		
-1 A –1 ATP + 1 B	R1	
-1 B +2 ATP +2 NADH +1 C	R2a	IF NOT (RPb)
-1 C –2 ATP –2 NADH +1 B	R2b	
-1 B +1 F	R3	
-1 C +1 G	R4	
-1 G +0.8 C +2 NADH	R5a	IF NOT (RPo2)
-1 G +0.8 C +2 NADH	R5b	IF RPo2
-1 C +2 ATP +3 D	R6	
-1 C –4 NADH +3 E	R7	IF NOT (RPb)
-1G –1 ATP – 2 NADH +1 H	R8a	IF NOT (RPh)
+1 G +1 ATP +2 NADH –1 H	R8b	
-1 NADH –1.02 +1 ATP	Rres	IF NOT (RPo2)
Transport Processes		
-1 Carbon1 +1 A	Tc1	
-1 Carbon2 +1 A	Tc2	IF NOT (RPc1)
-1 Fext +1 F	Tf	
-1 D +1 Dext	Td	
-1 E +1 Eext	Te	
-1 Hext +1 H	Th	
-1 Oxygen +1 O2	To2	
Maintenance/Growth Processes		
-1 C –1 F –1 H –10 ATP +1 Biomass	Growth	
Regulatory Proteins		
	RPo2	IF NOT (Oxygen)
	RPc1	IF Carbon1
	RPh	IF Th
	RPb	IF R2b

Table 5 provides parameters used to generate the plots in Figure 8A.

Parameters		
Initial Conditions		
$[Biomass]_0$ (g/L)	0.003	Estimated
$[Glucose]_0$ (mM)	10.4	Estimated
$[Acetate]_0$ (mM)	0.3	Estimated
Strain-specific parameters		
Protein synthesis/degradation delay (hrs)	0.4	Estimated
Biomass scaling factor	1.3	Varma and Palsson, 1994
*Uptake rate constraints (mM/(gDCW*hr))*		
Glucose	10.5	Varma and Palsson, 1994
Acetate	2.5	Estimated
Oxygen	15.0	Varma and Palsson, 1994

Table 6 provides the parameters used to generate figure 9A.

Parameters		
Initial Conditions		
$[Biomass]_0$ (g/L)	0.002	Estimated
$[Glucose]_0$ (mM)	10.5	Estimated
Strain-specific parameters		
Protein synthesis/degradation delay (hrs)	0.4	Estimated
Biomass scaling factor	1.3	Varma and Palsson, 1994
*Uptake rate constraints (mM/(gDCW*hr))*		
Glucose	18.5	Varma and Palsson, 1994
Oxygen	0.0	Varma and Palsson, 1994

A6

Table 7 provides the parameters used to generate the plots of Panel 10A.

Parameters		
Initial Conditions		
$[Biomass]_0$ (g/L)	0.011	Estimated
$[Glucose]_0$ (mM)	1.6	Estimated
$[Lactose]_0$ (mM)	5.8	Estimated
Strain-specific parameters		
Protein synthesis/degradation delay (hrs)	0.5	Estimated
Unconstraining [*lacZ*] (mmol/gDW)	0.0274	Estimated
Constraining [*lacZ*] (mmol/gDW)	0.0015	Estimated
*Uptake rate constraints (mM/(gDCW*hr))*		
Glucose	6.5	Estimated
Acetate	3.0	Estimated
Oxygen	15.0	Estimated

A6 Cont'd